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Original Contribution

Redox cycling of 9,10-phenanthraquinone to cause oxidative stress is terminated through its monoglucuronide conjugation in human pulmonary epithelial A549 cells

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Abstract

9,10-Phenanthraquinone (PQ), a component of airborne particulate matter, causes marked cellular protein oxidation and cytotoxicity through a two-electron reduction to 9,10-dihydroxyphenanthrene (PQH_2), which is associated with the propagation of reactive oxygen species (K. Taguchi et al., *Free Radic. Biol. Med.* 43:789–799, 2007). In the present study, we explored a biotransformation pathway for the detoxification of PQ. Exposure of human pulmonary epithelial A549 cells to PQ resulted in a time-dependent appearance of an unknown metabolite in the medium that was identified as the monoglucuronide of PQH_2 (PQHG). Whereas a variety of isozymes of uridine 5′-diphosphate glucuronosyltransferase (UGTs) are responsible for PQHG formation, UGT1A10 and UGT1A6 were particularly effective catalysts for glucuronide conjugation. In cell-free systems, PQ exhibited a rapid thiol oxidation and subsequent oxygen consumption in the presence of dithiothreitol, whereas PQHG did not. Unlike the parent compound, PQHG completely lost the ability to oxidize cellular proteins and cause cell death in A549 cells. In addition, deletion of the transcription factor Nrf2 decreased PQHG formation and increased PQ-mediated toxicity of mouse primary hepatocytes. Thus, we conclude that PQHG is a metabolite of PQ, generated through PQH_2, that terminates its redox cycling and transports it to extracellular space.

Keywords: Redox activity; Glucuronidation; 9,10-Phenanthraquinone; Detoxification; Free radicals

Airborne particulate matter (PM) has been suspected to be a cause of pulmonary and cardiovascular diseases [1,2] which have been reported to be oxidative stress-related disorders [3,4]. Small-diameter PM [5] readily access lung tissue and enter the circulation to initiate the induction of oxidative stress, but the causative chemical species have not been identified. One compound, 9,10-phenanthraquinone (PQ), which has been identified as a PM component [6,7], has been of particular interest to us because of its chemical and toxicological properties [8–10]. This quinone is produced from phenanthrene by oxidation in air [11] or by metabolic activation in cellular systems [12] and Baulig

Abbreviations: DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PM, particulate matter; PQ, 9,10-phenanthraquinone; $PQ^{\cdot-}$, 9,10-phenanthraquinone semiquinone radical; PQH_2, 9,10-dihydroxyphenanthrene; PQHG, monoglucuronide of PQH_2; ROS, reactive oxygen species; SAL, D-saccharic acid 1,4-lactone; SOD, superoxide dismutase; UDPGA, uridine 5′-diphosphate glucuronic acid; UGT, uridine 5′-diphosphate glucuronosyltransferase.

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et al. [13] have suggested that metabolites of phenanthrene contribute to the formation of reactive oxygen species (ROS) in human airway epithelial cells. PQ, in the presence of a reducing agent, generates ROS in a redox cycling process by enzymatic and nonenzymatic reactions [8,10]. The former is by one- or two-electron reducing enzymes using NAD(P)H and the latter is by dithiols.

Two-electron reduction of quinones to hydroquinones is basically thought to be a detoxification reaction [14]. However, some hydroquinones are fairly unstable. The highly reactive hydroquinones associated with redox cycling [15] require phase II enzymes for conjugation, and the conjugates are then transported to extracellular space to reduce their intracellular concentration. We have recently shown that a two-electron reduction product of PQ, 9,10-dihydroxyphenanthrene (PQH_2), is redox active as well as PQ because of the disproportionation reaction of PQ and PQH_2, generating a semiquinone radical ($PQ^{\cdot -}$) that reacts readily with molecular oxygen to produce superoxide [16]. It was also found that NAD(P)H:quinone oxidoreductase 1 (NQO1) and aldoketo reductase (AKR) catalyze the reduction of PQ to PQH_2 [16] (see Fig. 7).

Except for these two-electron reduction enzymes, a variety of cytoprotective enzymes are regulated by a transcription factor, Nrf2, relating to phase II detoxification and antioxidation. In normal cells, Nrf2 is kept in the cytoplasm by Keap1 protein and degraded by the ubiquitination–proteasome system [17]. Thiol modification in Keap1 by electrophiles and ROS accumulates Nrf2 in the nucleus and transcriptional activation occurs. Recently, it was reported that some cancer cells constitutively activate Nrf2 by a point mutation in the *KEAP1* gene [18]. Nrf2 activation helps cancer cells resist anti-cancer drugs via expression of multidrug resistance proteins (MRPs), which are a kind of Nrf2 target protein.

Uridine 5′-diphosphate (UDP) glucuronosyltransferases (UGTs) are phase II enzymes that catalyze the conjugation of glucuronic acid with –OH, –SH, –COOH, and $–NH_2$ groups. UGTs play a critical role in the elimination of phenolic metabolites, such the phenols and quinols of polycyclic aromatic hydrocarbons. Thus, we hypothesized that PQ could undergo two-electron reduction and subsequent glucuronidation by NQO1/AKR and UGT isozymes to yield its glucuronide. This polar metabolite would be excreted into extracellular space by MRP transporters. The purpose of the present study was to elucidate the detoxification process of PQ in human pulmonary epithelial A549 cells. Because some UGT isozymes and MRPs are reported to be controlled by Nrf2 as well [19,20], we also examined the effects of Nrf2 deletion on the cellular toxicity of PQ with primary hepatocytes from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice.

Material and Methods

Material

PQ, superoxide dismutase (SOD), dimethyl sulfoxide (DMSO), ethoxyquin, monoclonal anti-dinitrophenyl (DNP) antibody, protease inhibitor cocktail, and D-saccharic acid 1,4-lactone (SAL) were from Sigma–Aldrich Co. (St. Louis, MO, USA); 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and dithiothreitol (DTT) were from Nacalai Tesque (Kyoto, Japan); β-glucuronidases type B-1 and H-2 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were obtained from commercial sources and were of the highest grade available. An AKR1C isozyme was purified from rabbit liver cytosol as described previously [16].

Enzyme preparation

ICR male mice (6–8 weeks of age) were given feed including 0.5% ethoxyquin (CLEA Japan, Tokyo, Japan), an inducer for AKR isozymes, for 2 weeks [21]. C57BL/6J ($Nrf2^{+/+}$) and Nrf2-knockout ($Nrf2^{-/-}$) male mice (6–8 weeks) were used.

Liver or lung tissue was homogenized in 9 volumes of 5 mM Tris–HCl (pH 7.5)–0.25 M sucrose–0.1 mM EDTA. The homogenate was centrifuged at 9000*g* for 20 min. The supernatant (9000*g* sup) from this step was again centrifuged at 105,000*g* for 60 min and the resulting supernatant, the cytosol fraction, was stored at −70 °C until use. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard [22].

Cells

Mouse primary hepatocytes were isolated according to the method described by Liang et al. [23] with slight modifications.

Human lung epithelial A549 cells were used as described in Sugimoto et al. [10]. After exposure of A549 cells to PQ (10 μM), a 100 μl aliquot of the medium was diluted with trichloroacetic acid (TCA) to a final concentration of 2.5%. This sample represented extracellular space. The cells were washed with Dulbecco's phosphate-buffered saline and then TCA was added to a final concentration of 2.5% in 1 ml. This sample represented intracellular space. These samples were centrifuged at 14,000*g* for 5 min at 4 °C to remove proteins and the supernatants used as extracellular and intracellular samples, respectively.

Identification and genotyping of somatic mutations in the human KEAP1 gene

The genomic sequence of human *KEAP1* was downloaded from the NCBI human genome database. Seven sets of PCR primers were designed to amplify the coding regions of the *KEAP1* gene. Direct sequencing of the PCR products was performed basically according to the method described by Yamamoto et al. [24]. Briefly, amplification of DNA from early passage A549 cell line was carried out using ExTaq Premix from Takara Mirus Biosciences (Madison, WI, USA) and 10 pmol of each primer. PCR products were directly sequenced after purification (QIAquick PCR purification kit; Qiagen). Sequence reactions were carried out using an ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit and genotyped by an ABI 3100 DNA sequencer. The sequence data were

downloaded, assembled, and analyzed to identify potential genetic alterations. All mutations were confirmed by sequencing in both directions.

Recombinant UGT

Infection of recombinant human UGT baculovirus into *Spodoptera frugiperda* 9 (Sf9) insect cells followed the Bac-to-Bac baculovirus expression system manual instructions (Invitrogen Corp., Carlsbad, CA, USA). A volume of 30 ml of log-phase Sf9 cells (1×10^6 cells/ml) was infected with 1 ml of recombinant human UGT baculovirus. After the cells were cultured at 27 °C for 72 h, the medium was centrifuged at 300*g* for 6 min at 4 °C. The pellets (Sf9 cells) were resuspended in 4 ml of 50 mM Tris–HCl (pH 7.4)–10 mM $MgCl_2$ (buffer A) with an ultrasonic generator. After the suspension was centrifuged at 43,000*g* for 20 min at 4 °C, the pellet was resuspended in 1 ml of buffer A–20% glycerol, to provide a recombinant UGT solution.

Biosynthesis of PQ-glucuronide

The reaction mixture (0.75 ml) consisted of 0.1 M Hepes (pH 7.6)–0.1 mM EDTA, 1 mM NADPH, 1 mM UDP glucuronic acid (UDPGA), 0.05% Brij58, 100 U/ml SOD, 9000*g* supernatant of ethoxyquin-treated mouse liver (0.119 mg/ml), and 50 μM PQ. The reaction was allowed to proceed at 25 °C for 3 h and was stopped by 10% TCA (0.25 ml, final concentration 2.5%), and the mixture was centrifuged at 14,000*g* for 5 min at 4 °C. For purification of the glucuronide, the supernatant (100 ml) was added to 200 ml of 0.5 M citrate buffer (pH 3.0) and fractionated on a Cosmosil 75C18-OPN (3.9× 2.5 cm, i.d., 19.1 cm^3) column at a flow rate of 1 ml/min. After the column was washed with 50 mM citrate buffer (pH 3.0) (100 ml), a metabolite was eluted with 30 ml of water and then methanol from 40 to 60% (200 ml). The collected fractions were concentrated by evaporation. After freeze-drying, the metabolite collected was recrystallized in dichloromethane.

HPLC

Detection of PQ and its metabolite was carried out on a YMC-Pack ODS-AM (250×4.6 mm, i.d., 5 μm particle size; YMC Co. Ltd., Kyoto, Japan) with a Shimadzu LC-10AT pump and SPD-10A UV–Vis detector (Kyoto, Japan). Elution was accomplished with acetonitrile/1% acetic acid (3/2, v/v) at a flow rate of 1 ml/min. Detection was performed at 255 nm. Peak height was determined by a Chromatocorder 11 (System Instruments Co. Ltd., Tokyo, Japan).

Mass spectrometry

A JMS-700T equipped with a fast atom bombardment (FAB) ion source (JEOL Ltd., Tokyo, Japan) was used as a mass spectrometer. High-resolution mass spectrometry also used the same equipment for atom composition of fragment ions.

Protein oxidation

Carbonylated proteins were detected as their DNPH derivatives by Western blotting according to the method of Levine et al. [25]. In the procedure, 1 volume of cell lysate in 6% SDS was incubated with 2 volumes of 20 mM DNPH dissolved in 10% trifluoroacetic acid for 30 min at room temperature and then neutralized in 2 M Tris–30% glycerol–15% 2-mercaptoethanol, bromophenol blue. Western blotting was carried out using a DNP antibody (×1000 dilution), biotinylated mouse anti-mouse IgE antibody (×4000 dilution), and streptavidin–HRP conjugate (×4000 dilution).

MTT assay

The MTT assay was used to estimate the cytotoxicity of PQ or PQHG [26]. Proliferating A549 cells were exposed to PQ or PQHG (1–50 μM) for 12 h in 96-well microtiter plates and then treated with 5 mg/ml MTT (1/20, v/v) for 4 h at 37 °C. After removal of the medium, DMSO (100 μl/well) was added to dissolve the cells. Absorbance at 540 nm was measured by the ImmunoMini NJ-2300 plate reader (Nippon InterMed, Tokyo, Japan).

Thiol consumption

The thiol exchange reaction of DTNB was performed according to the method of Sedlak and Lindsay [27]. The reaction mixture (1 ml) consisted of 0.1 M Hepes (pH 7.6)–3 mM EDTA, 0.5 mM DTT, and PQ, PQH_2, or PQHG (5 or 10 μM). The reaction was allowed to proceed for 10 min at 37 °C, then 0.1 ml of the reaction mixture was diluted with Tris–HCl (pH 8.9)–13 mM EDTA, to a final concentration of 0.26 M. At 2 min after addition of DTNB (final concentration 0.16 mM), the absorbance at 412 nm was measured. DTT (0–0.5 mM) was used to generate a standard curve. A molar extinction coefficient of $\lambda_{412}=13.6\ mM^{-1}\ cm^{-1}$ was used to calculate thiol content.

Oxygen consumption

Cells were collected in 200 μl of a mixture of 20 mM Hepes–0.25 M sucrose–5 mM EDTA (pH 7.5), and protease inhibitor cocktail (1/100 olume). After ultrasonication, the suspension was centrifuged at 14,000*g* for 5 min at 4 °C. The supernatant was used as a cell lysate.

Results

The KEAP1 gene in human pulmonary epithelial A549 cells

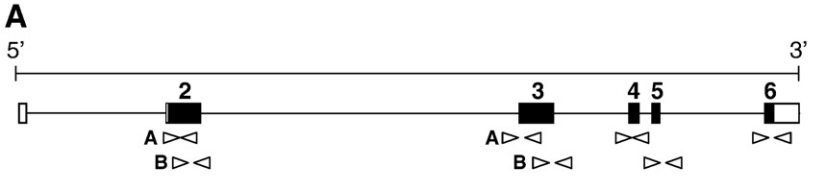
Quinones are generally metabolized to two-electron reduction compounds. Recently, we reported that the two-electron reduction of PQ propagates ROS production as much as the parent compound [16]. We assumed that PQ needs to be further converted to an ultimate metabolite to be excreted to the extracellular space. Our hypothesis is that PQH_2 is conjugated with one or two glucuronic acid(s) to terminate redox cycling (see

Fig. 2A) and that the glucuronides are excreted through transporters such as MRPs. It is known that the enzymes are inclusively regulated by Nrf2 [20]. In our previously reported paper, we found that heme oxygenase-1, a Nrf2 target protein, is innately induced in A549 cells in the absence of stimuli such as heme or heavy metals [10]. We suspected that the Keap1–Nrf2 system is genetically disrupted to lead to the constitutive activation of Nrf2 target genes in A549 cells.

Seven sets of PCR primers were designed to amplify the coding regions of the *KEAP1* gene (Fig. 1A). The A549 cell has a point mutation in its *KEAP1* gene, a G → T substitution at position +997 (Fig. 1B). This position corresponds to G333C in a double-glycine-rich region comprising six Kelch motifs that binds to the Neh2 domain of Nrf2. This single mutation exerts the nuclear accumulation of Nrf2 [18], resulting in the upregulation of a variety of phase II detoxification enzymes and transporters including NQO1, UGTs, and MRPs. These Nrf2 target proteins might play an important role in detoxification of PQ. Therefore, we examined a detoxification product of PQ using A549 cells.

Identification of a metabolite of PQ in extracellular space

As shown in Fig. 2A, two enzymatic reactions are required for glucuronide conjugation of PQ; one is two-electron reduction of PQ to PQH_2 by NAD(P)H-dependent reductases such as NQO1 and AKR, and the other is glucuronidation of PQH_2 with UDPGA by an UGT isozyme. To identify such a metabolite, we first synthesized the glucuronide conjugate using enzymes available in liver supernatant as described under Material and methods. The reaction mixture contained SOD to diminish superoxide-dependent oxidation of PQH_2 to PQ [16]. The mass spectrum of the purified reaction product showed that it was the monoglucuronide of dihydroxyphenanthrene (PQHG), whose molecular weight is 386 (Fig. 2B, graph a). Peaks at *m/z* 209, 385, and 407 corresponded to $[PQH_2–H]^-$, $[PQHG–H]^-$, and



Name	Sequence
hKeapE2AF	5'-tgtaaaacgacggccagtAGGTGCTGGCCCAGTCCCAAC-3'
hKeapE2AR	5'-caggaaacagctatgaccGCAGCCCGTTGGTGAACATGG -3'
hKeapE2BF	5'-tgtaaaacgacggccagtCCACAAGGTGGTGCTGGCCTC-3'
hKeapE2BR	5'-caggaaacagctatgaccGGTCCTTCTCCTGACACTGCC -3'
hKeapE3AF	5'-tgtaaaacgacggccagtGGGTGACTGGAGAGTCAGCC-3'
hKeapE3AR	5'-caggaaacagctatgaccTGAGCGACTGTCGGAAGTAG -3'
hKeapE3BF	5'-tgtaaaacgacggccagtACAAGCCCACGCAGGTGATGC-3'
hKeapE3BR	5'-caggaaacagctatgaccTCAGTGTCTTGGGACTTGCCAGG -3'
hKeapE4F	5'-tgtaaaacgacggccagtCAGCTATAATGGCCATTGTC-3'
hKeapE4R	5'-caggaaacagctatgaccAGGATGGTAGGGGGTGTTCC -3'
hKeapE5F	5'-tgtaaaacgacggccagtGACCCCCAG AGTCACCTTCTCT-3'
hKeapE5R	5'-caggaaacagctatgaccATGGGCTAGTCAGGACTCTTCC -3'
hKeapE6F	5'-tgtaaaacgacggccagtGTGACAGGTGGTGACCATCC-3'
hKeapE6R	5'-caggaaacagctatgaccAATGATACTCCCCATTGGACTG -3'

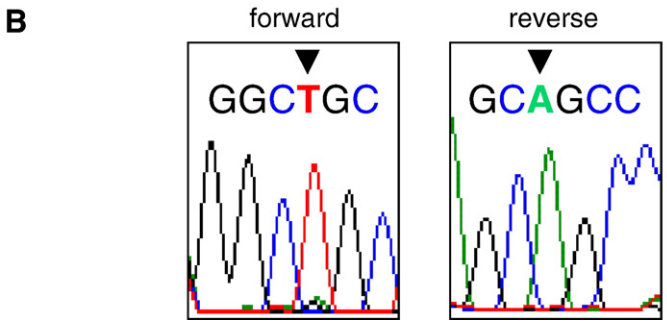


Fig. 1. *KEAP1* mutation in human pulmonary epithelial A549 cells. (A) Sequences of oligonucleotides used for sequencing the coding region of the human *KEAP1* gene. Uppercase letters represent the sequences derived from the human *KEAP1* gene locus, whereas lowercase letters represent synthetic sequences. (B) Detection of polymorphism in *KEAP1* coding region sequence of A549 cell. Sequence chromatographs from the *KEAP1* coding region of A549 cells showing a G to T substitution at position +997 are presented.

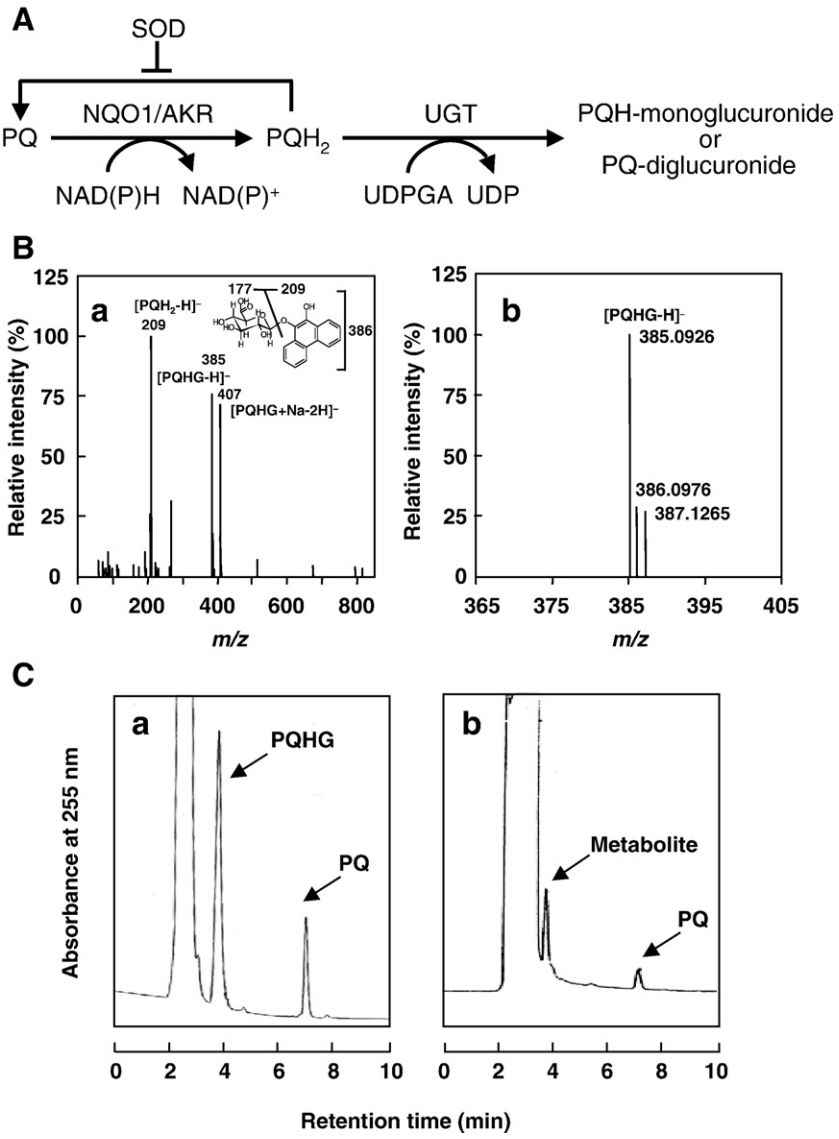


Fig. 2. (A) Predicted metabolic reactions of PQ to an ultimate metabolite. (B) FAB–MS analysis of a glucuronide of PQH_2 in negative ion mode (a) and negative ion mode/high resolution (b). Molecular weight: PQ, 208; PQH_2, 210; PQHG, 386. (C) Chromatograms of PQHG (a) and an unknown metabolite excreted from A549 cells exposed to PQ (10 μM) (b). (a) 9000*g* supernatant from mouse liver (1.18 mg protein) was incubated with 1 mM NADPH, 1 mM UDPGA, 100 U/ml SOD, 0.05% Brij58, and 50 mM PQ in 0.1 M Hepes (pH 7.6), 0.1 mM EDTA, at 25 °C for 1 h. The reaction was stopped by addition of 2.5% TCA. (b) Medium after A549 cells were exposed to 10 mM PQ for 8 h with 2.5% TCA addition.

$[PQHG+Na-2H]^-$ and the high-resolution mass spectrum showed a peak at *m/z* 385.0926, corresponding to $[PQHG-H]^-$ (Fig. 2B, graph b). However, no diglucuronide conjugate of PQH_2 was detected in the mixture.

The HPLC retention times of authentic PQHG and PQ under the conditions used were 3.8 and 6.9 min, respectively (Fig. 2C). When a portion of the cell medium at 8 h after exposure of A549 cells to PQ was injected into HPLC, there was an unknown metabolite with the same retention time as authentic PQHG. As shown in Fig. 3A, PQ was rapidly taken up into the intracellular space after exposure of A549 cells and then an increase in the metabolite was accompanied by a decrease in PQ in the extracellular space (Fig. 3A, graph b). Little appreciable metabolite was detected in the cells throughout exposure to PQ (Fig. 3A, graph a).

To confirm whether the polar metabolite of PQ is identical to PQHG, the cell medium, after exposure to PQ, was incubated with two β-glucuronidases, type B-1 from bovine liver and type H-2 from *Helix pomatia,* because these enzymes catalyze the hydrolysis of glucuronide conjugates to glucuronic acid and its aglycon. We then monitored levels of the unknown metabolite, which was tentatively identified as PQHG on the basis of subsequent experiments. Treatment of control cellular medium containing authentic PQHG with B-1 and H-2 caused a reduction in PQHG levels and an associated increase in PQ levels (Fig. 3B, graph a). When cellular media from A549 cells exposed to PQ were treated in the same manner, we found a decrease in unknown metabolite levels, which was accompanied by increased PQ levels (Fig. 3B, graph b). This relationship was found to be dependent on the concentration of β-glucuronidases (data not shown). Collectively,

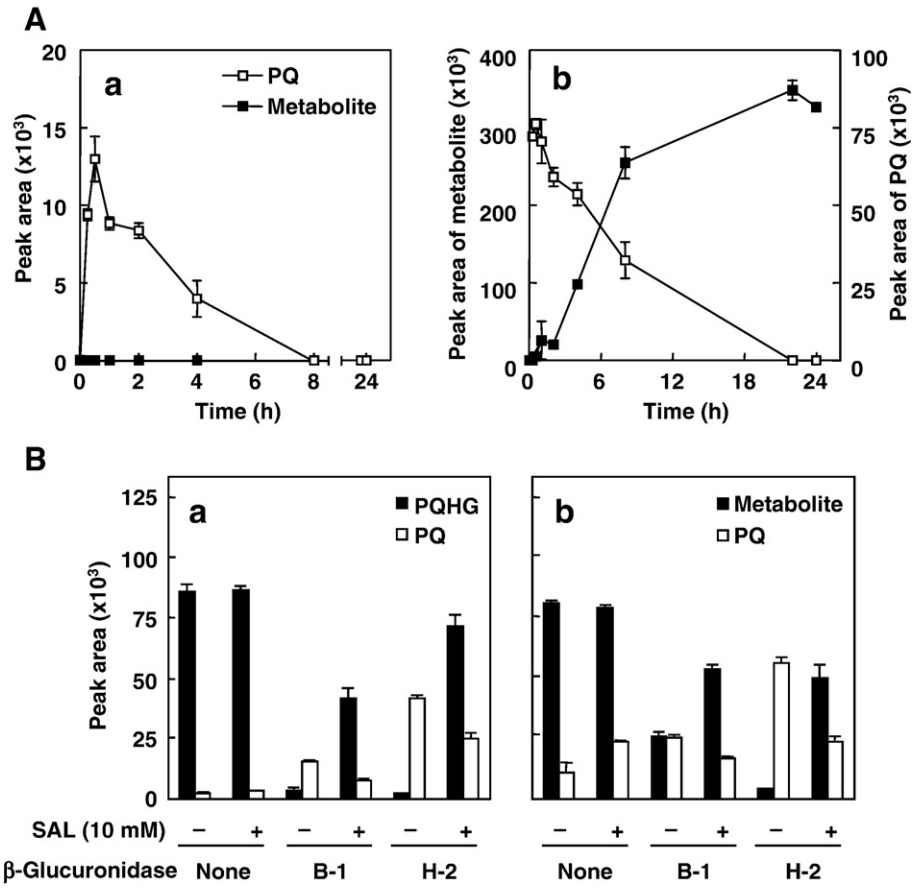


Fig. 3. Identification of an excreted metabolite as a glucuronide. (A) Time-dependent changes in PQ and an unknown metabolite in intracellular (a) and extracellular space (b). A549 cells were exposed to PQ (10 μM) for the indicated times. Each value is the mean±SD (n=3). (B) Effect of a β-glucuronidase inhibitor, SAL, on PQHG and an unknown metabolite. β-Glucuronidase type B-1 or H-2 (5000 U/ml) was incubated with 10 μM PQHG in the medium of A549 cells (a) or medium of A549 cells exposed to PQ (10 μM) for 16 h (b) in the presence or absence of 10 mM SAL at 37 °C for 30 min. The reaction was stopped by addition of 2.5% TCA. Each value is the mean±SD (n=3).

these results are consistent with the notion that PQ is metabolized ultimately to PQHG, which is transported into extracellular space.

UGT isozyme specificity for PQHG formation

Fifteen human UGTs catalyze the addition of glucuronic acid(s) to aglycons [28]. As shown in Fig. 4, a variety of UGT isoforms are responsible for glucuronidation of PQ via PQH_2 among 8 recombinant UGTs examined. For example, UGT1A10 and 1A6 catalyzed PQHG formation efficiently, whereas 1A4 and 2B17 were poor catalysts for the glucuronidation.

Assessment of PQHG on redox activity and cell damage

Considering the chemical structure of PQHG, we presumed the possibility that this polar metabolite would be devoid of redox activity and thus exhibit little cellular toxicity. To address this issue, we compared the redox activity of the two compounds, as determined by DTT thiol oxidation and oxygen consumption. We also compared their cellular toxicity, evaluated by the MTT assay, and their ability to induce cellular protein oxidation. PQ will catalyze the reduction of oxygen by dithiols, resulting in the oxidation of the thiol and reduction of oxygen to superoxide and other ROS [8]. In contrast, PQHG was unable to carry out the reaction; there was a loss neither of thiol nor of oxygen under conditions under which PQ caused both (Figs. 5A and B). Consistent with these findings, PQ, but not

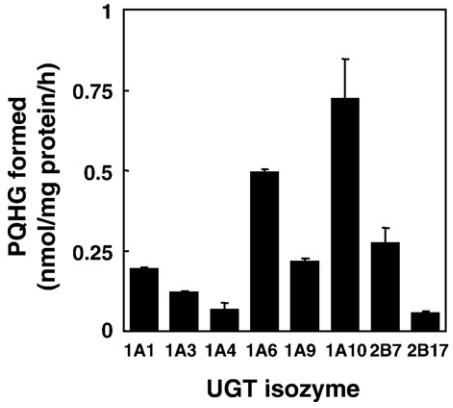


Fig. 4. Specificity of UGT isozymes in formation of PQHG. The reaction mixture consisted of 60 mM Tris–HCl (pH 7.4)–4.8 mM $MgCl_2$, 1 mM NADPH, 1 mM UDPGA, 100 U/ml SOD, 2.98 mU AKR1C isozyme, UGT isozyme as indicated, and 50 μM PQ. The mixture was incubated at 25 °C for 1 h. Each value is the mean±SD (n=3).

PQHG, caused extensive oxygen consumption during reaction with DTT or exposure to cell lysates of A549 (Fig. 5B). Although A549 cells were sensitive to PQ with a LD_{50} value of 2.8 μM, PQHG exhibited no cytotoxicity up to concentrations of 50 μM (Fig. 5C). Furthermore, PQHG was without effect of protein oxidation in A549 cells, whereas PQ exhibited results typical for oxidative modification of the cellular proteins (Fig. 5D).

Involvement of Nrf2 in detoxification of PQ

If PQHG were an ultimate detoxification metabolite of PQ, steady-state levels of enzymes that participate in PQHG formation would affect cellular toxicity caused by PQ. It is well recognized that downstream genes of the transcription factor Nrf2 such as *NQO1,* some *AKRs, UGTs,* and *MRPs* play an important role in the detoxification of xenobiotics [19,20]. Thus, we speculated that deletion of Nrf2, leading to downregulation of these proteins, could accelerate PQ-mediated cytotoxicity. To explore such a possibility, we used enzyme preparations and primary hepatocytes from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. As shown in Fig. 6A, formation of PQH_2 and PQHG during metabolism of PQ in the presence of NADPH and/or UDPGA by cytosolic and 9000*g* supernatant fraction of liver and lung from $Nrf2^{-/-}$ mice was significantly lower than in those from $Nrf2^{+/+}$ mice. Under these conditions, the LD_{50} value in primary hepatocytes from $Nrf2^{-/-}$ was 10.75 μM, which was lower than that from $Nrf2^{+/+}$, 22.13 μM (Fig. 6B).

Discussion

PQ is one of the oxidized forms of phenanthrene, which is produced chemically and enzymatically. Monohydroxyphenanthrenes (1-, 2-, 3-, 4-, or 9-hydroxy) have been extensively

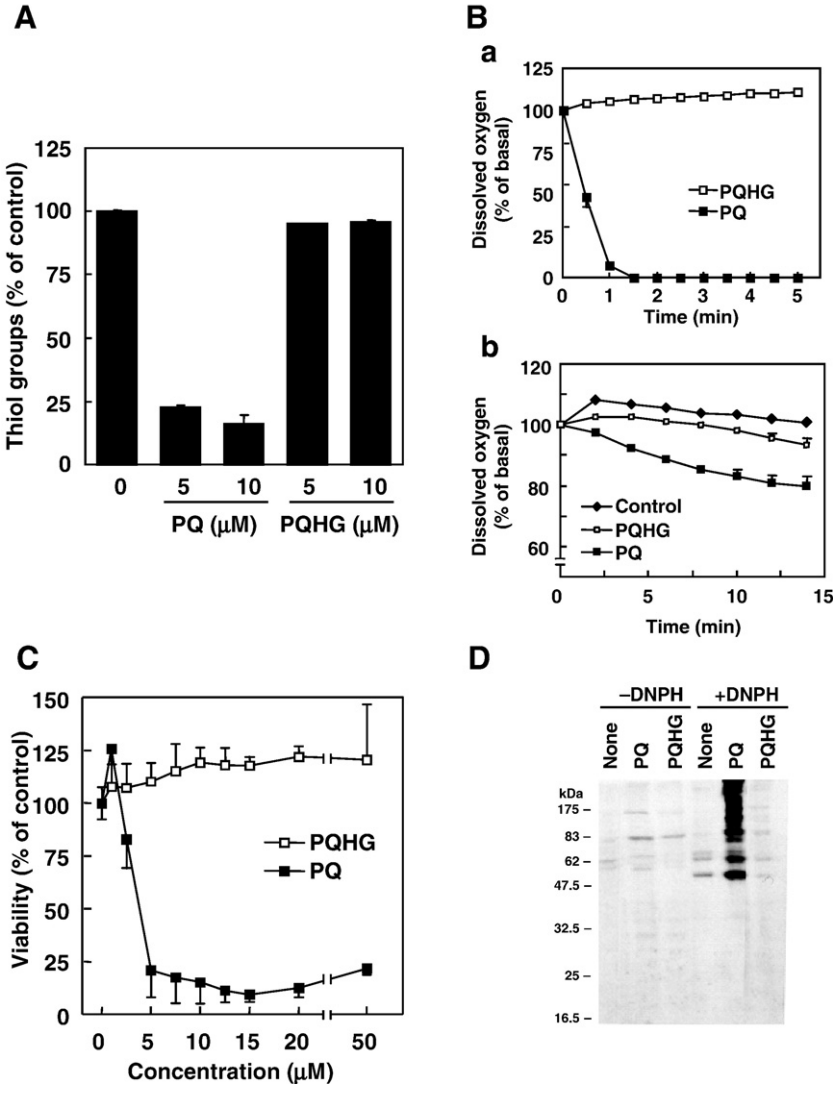


Fig. 5. Correlation between redox activity and cell damage. (A) Thiol consumption. The reaction mixture consisted of 0.5 mM DTT, test compound (PQ or PQHG; 5 or 10 μM), and 0.1 M Hepes (pH 7.6)–3 mM EDTA, and reacted at 37 °C for 10 min. Each value is the mean ± SD (n = 3). (B) Oxygen consumption with DTT (a) or A549 cell lysate (b). (a) The reaction mixture consisted of 0.5 mM DTT and test compound (PQ or PQHG; 1 or 5 μM). Each value is the mean ± SD (n = 3). (b) The reaction mixture consisted of A549 cell lysate (0.1 mg protein), 0.2 mM NADPH, a compound (PQ or PQHG), and 0.1 M Hepes (pH 7.6)–3 mM EDTA. Each value is the mean ± SD (n = 1 for control, n = 2 for PQHG, n = 3 for PQ). (C) Cell viability. A549 cells were exposed to PQ or PQHG (0–50 μM) for 12 h. Each value is the mean ± SD (n = 8). (D) Protein oxidation. A549 cells were exposed to PQ or PQHG (10 μM) for 12 h.

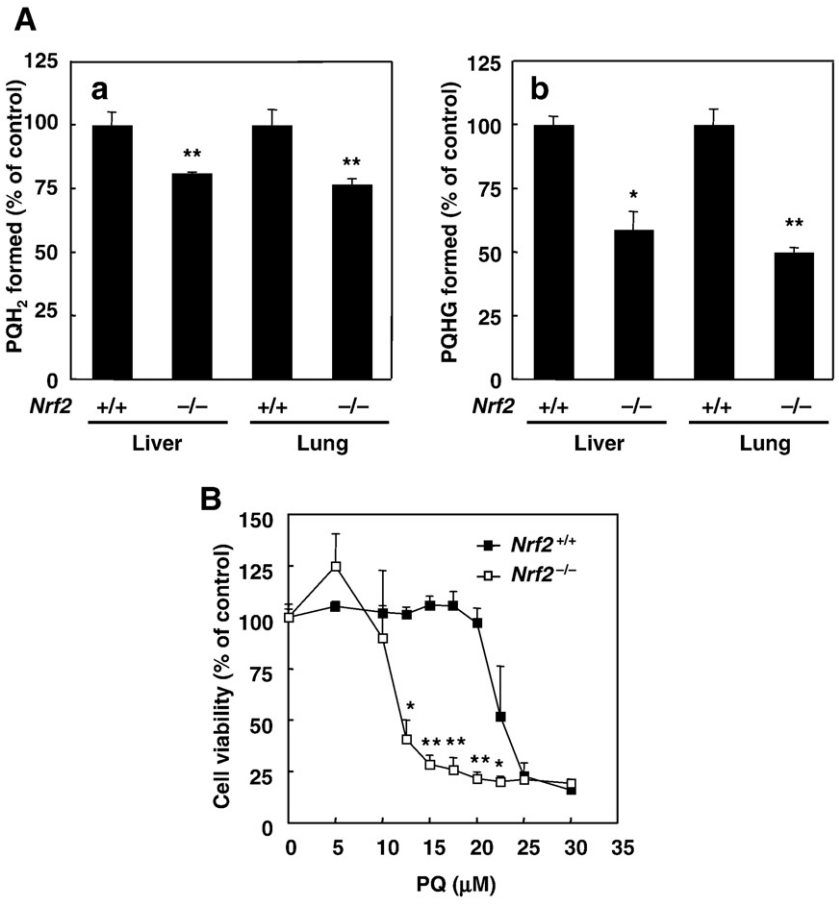


Fig. 6. Involvement of Nrf2 in detoxification of PQ. (A) (a) Formation of detoxification products of PQ. Liver or lung cytosol from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice (10 μg protein) was incubated with 0.2 mM NADPH, SOD (100 U/ml), and 5 μM PQ in 0.1 M Hepes (pH 7.6)–0.1 mM EDTA, at 25 °C for 3 min. PQH_2 formed was determined as its stable diacetoxy derivative by HPLC. After reaction, the mixture was incubated with acetic anhydride (20 μl) at 90 °C for 5 min. Enzyme activities of PQH_2 formation from $Nrf2^{+/+}$ mice were 31.19±1.53 (liver) or 31.52±1.88 (lung) nmol/mg/min. (b) Liver or lung 9000*g* supernatant from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice (1.156 mg protein) was incubated with 1 mM NADPH, 1 mM UDPGA, 100 U/ml SOD, 0.05% Brij58, and 50 μM PQ in 0.1 M Hepes (pH 7.6)–0.1 mM EDTA, at 25 °C for 1 h. The reactions were stopped by addition of 2.5% TCA. After centrifugation at 14,000 *g* for 5 min, the supernatant (40 μl) was applied to HPLC. Enzyme activities of PQHG formation from $Nrf2^{+/+}$ mice were 18.32±0.61 (liver) and 1.34±0.67 (lung) nmol/mg/h. Each value is the mean±SD ($n=3$). $^{**}p<0.01$; $^{*}p<0.05$. (B) Cell viability. Primary hepatocytes from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice were exposed to PQ for 12 h. $Nrf2^{+/+}$, $LD_{50}=22.13$ μM; $Nrf2^{-/-}$, $LD_{50}=10.75$ μM. Each value is the mean±SD ($n=8$).

examined as in vivo metabolites of phenanthrene in urine [29,30]. However, actual metabolites in urine have never been identified, because the urine samples examined were treated with β-glucuronidase and arylsulfatase before HPLC analysis. Moreover, no one has examined the in vivo metabolites of PQ, although phenanthrene is oxidized to PQ by a series of cytochrome P450 s and AKRs [12,31]. Glucuronidation of PQH_2 is expected to terminate the redox activity as part of the detoxification of PQ. In this study, we have identified the glucuronide and showed that the redox cycling properties of PQ are terminated after reduction and glucuronidation.

Two chemical properties of quinones are well known [12]: (1) Michael addition and (2) redox cycling. We have previously reported that PQ carries out nonenzymatic redox cycling with dithiols [8]. Furthermore, enzymatic two-electron reduction of PQ also initiates redox cycling after disproportionation of PQ and its two-electron reducing product, PQH_2 [16]. This means that two-electron reduction of PQ by itself is not a detoxification reaction but one that has the potential for protein oxidation. SOD is effective in suppressing this redox cycling, because superoxide acts as a mediator of oxidation from PQH_2 to $PQ^{\bullet-}$.

Here, we report that PQ is rapidly taken up by cells, decreasing extracellular PQ and increasing extracellular levels of a metabolite in a time-dependent manner (Fig. 3A). The extracellular metabolite was identified as a monoglucuronide of PQH_2, PQHG, by decomposition of the metabolite by β-glucuronidase (Fig. 3B) and by the coincidence with the retention time of the biosynthesized preparation (Fig. 2B). The PQ in cells would be reduced enzymatically by one or two electrons to $PQ^{\bullet-}$ or PQH_2, as some in vitro experiments indicate. $PQ^{\bullet-}$ and PQH_2 carry out redox cycling reactions to produce high levels of superoxide. PQH_2 does, however, serve as an intermediate in the detoxification of PQ to PQHG, an ultimate metabolite conjugated with UDPGA by UGTs. Once formed, the PQHG is rapidly exported to extracellular space because it is undetectable in intracellular space as shown in Figs. 3A and 7.

The UGT family catalyzes the glucuronidation of the glycosyl group of a nucleotide sugar to an aglycon at a nucleophilic

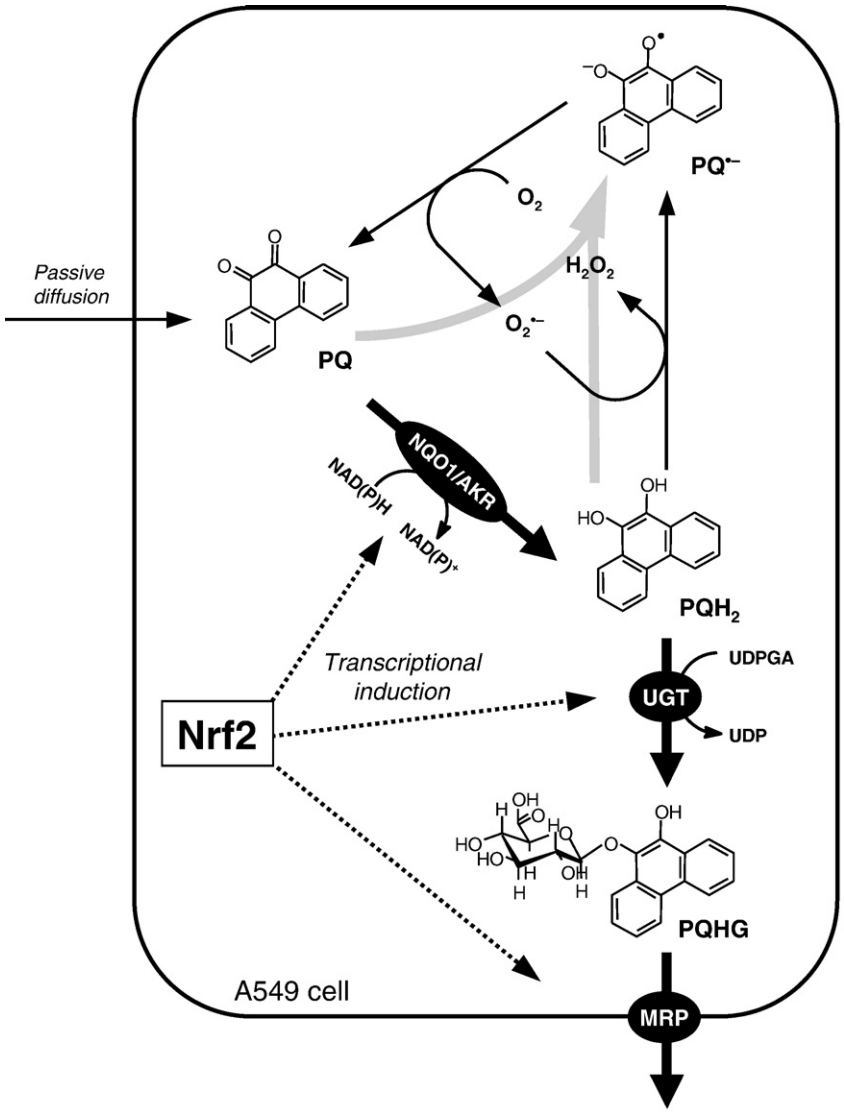


Fig. 7. Redox cycling of PQ is terminated by glucuronidation. PQ, 9,10-phenanthraquinone; $PQ^{\bullet-}$, 9,10-phenanthraquinone semiquinone radical; PQH_2, 9,10-dihydroxyphenanthrene; PQHG, PQH monoglucuronide; H_2O_2, hydrogen peroxide; AKR, aldoketo reductase; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, NF-E2-related factor 2; $O_2^{\bullet-}$, superoxide; UGT, uridine 5′-diphosphate glucuronosyltransferases; UDP, uridine 5′-diphosphate; UDPGA, uridine 5′-diphosphate glucuronic acid.

functional group of oxygen (hydroxyl or carboxylic acid groups), nitrogen (amines), sulfur (thiols), and carbon [32]. To date, over 100 different UGT gene products have been described from several different species [33]. Each isozyme has relatively broad substance specificities to drugs, carcinogens, and endogenous substrates. We examined the contribution of 8 different isozymes to the glucuronidation of PQH_2. UGT1A6 and 1A10, exhibiting high activity for glucuronidation of PQH_2, are major UGT isozymes in the liver and gastrointestinal tract, respectively. Substrate specificity of UGT1A6 is limited to small planar phenols [34].

PQHG is passed out to the extracellular space, probably through a transporter(s), i.e., MRP. In contrast to PQH_2, which has a redox-active potency as well as PQ [16], PQHG has neither cytotoxicity nor redox activity (Fig. 5). It has been reported that glucuronidation of phenols and quinols of toxic polycyclic aromatic hydrocarbons plays a protective role against their toxicity [35–37]. The results of this study demonstrate that the monoglucuronide of PQH_2 is devoid of redox activity, so although PQ and its secondary metabolite PQH_2 cause oxidative stress, formation of PQHG results in detoxification and rapid elimination from the cell. Recently, it has been reported that Nrf2 is constitutively activated in human pulmonary epithelial A549 cells [18]. A549 cells would be proper to examine the detoxification of PQ. Nrf2 is a transcription factor that upregulates the transcriptional expression of some genes with the antioxidant-responsive element/electrophile-responsive element. Some two-electron reductases (e.g., NQO1); UGT isozymes, which catalyze a consecutive detoxification metabolism of PQ; and MRPs are known to be Nrf2 target proteins, suggesting that deletion of Nrf2 will affect the formation of PQH_2 and PQHG from PQ. Consistent with this hypothesis, levels of these metabolites of PQ in the enzyme preparation from $Nrf2^{+/+}$ mice were significantly lower than in those from $Nrf2^{-/-}$ mice (Fig. 6A). Although PQH_2 is still a redox-active metabolite, two-electron reduction of PQ to PQH_2 is an obligatory pathway for biotransformation of

PQHG to lose redox activity (see Fig. 7). A possible explanation for the different sensitivities of primary hepatocytes from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice to PQ (Fig. 6B) is that it is due to the metabolic capacity to produce PQHG from PQ via PQH_2 by Nrf2-dependent phase II enzymes.

In preliminary studies, we have found that, whereas Nrf2 is constitutively active in A549 cells, PQ has the ability to activate Nrf2 in another cell (K. Taguchi et al., unpublished data), possibly by the ROS-based oxidation of the Keap1 thiol. The protein Keap1 is a suppressor of Nrf2 and modification of its thiol releases Nrf2 from its complex, allowing it to enter the nucleus. Thus, we speculate that such cells are capable of increasing resistance to subsequent PQ exposure by further activation of Nrf2.

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